International Journal of Bio-Technology and Research (IJBTR)
ISSN(P): 2249-6858; ISSN(E): 2249-796X
Vol. 4, Issue 3, Jun 2014, 1-8


TRANS STELLAR
• Journal Publications • Research Consultancy

# STUDY ON EFFICACY OF TWO STRAINS (ATCC 15834 AND MTCC 532) OF *AGROBACTERIUM RHIZOGENES* ON HAIRY ROOT INDUCTION OF *WITHANIA SOMNIFERA*

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## ABSTRACT

*Withania somnifera* L. Dunal is an important medicinal plant of the family solanaceae. Efficacy of two *Agrobacterium rhizogenes* strains ATCC 15834 and MTCC 532 was checked for transformation frequency, number of root induction and lateral branching in roots were observed in the present study. It was observed that even though using same type of explants (shoot tip) the two strains showed variation in each study. Out of these two strains ATCC15834 was dominated over MTCC 532 in every respect. The present study was showed a detailed result of hairy root induction of *Withania somnifera*. Strain ATCC15834 showed maximum 91.29 ± 1.75 % of transformation frequency, 11.15 ±0.30 number of roots /explants and 46.09 ± 0.16 number of lateral branching in roots /explants in 3 hours of co-culture period while MTCC 532 gave 85.41 ± 2.08 % of transformation frequency, 08.27 ± 0.35 number of roots / explants and 35.12 ± 0.23 number of lateral branching in roots /explants in 4 hours of co-culture period. After successful achievement of genetic transformation, for the first time in the present study it was shown that hairy roots induce directly from the sub cultured part of the plant (Leaf, Shoot tip, Stem segments and from the roots also)

**KEYWORDS:** Hairy Root, *Withania*, Tropine, T-DNA, Transformation Frequency

## INTRODUCTION

*Withania somnifera* commonly known as Ashwagandha, is an important medicinal plant in indigenous medicinal system of India from ancient time. Resaerches supports its poly pharmaceutical use confirming antioxidant, anti inflammatory, immune modulating and anti stress properties. It is described as an herbal tonic and health food in Vedas and considered as Indian Ginseng in traditional Indian system of medicine). It is generally used as anti-inflammatory, anticancer, anti stress, and immune modulator, adaptogenic, central nervous system, endocrine and cardio-vascular activities respectively (Bhattacharya et al., 1997a; Rai, et al., 2003; Ahmad, et al., 2005). The active principles as Withanine, somniferine, Withanine, somenine and tropine along with withaferine accumulates in roots as secondary metabolites which are of pharmaceutical interest necessitates uprooting of the whole plant for harnessing the compounds *which* brings the plant under threatened category.

*Agrobacterium rhizogenes* is a gram negative soil bacterium causing hairy root disease in dicot plants during the course of infection the bacteria transfers a part of its plasmid DNA (T- DNA) into the plant genome which makes the bacteria a natural genetic engineer. Hairy roots are characterized by rapid growth and extensive branching in growth regulator free medium. They exhibit genetic stability & have capability of synthesizing secondary metabolites. Researches show that these hairy roots produce secondary metabolites larger in amount than normal roots most *Agrobacterium* mediated transformations are carried out using in vitro tissue culture.

## MATERIAL AND METHODS

Aseptic cultures of withania somnifera was established sterile shoot tips of withania were used as explants for genetic transformation. Strain ATCC 15834 and MTCC 532, used for hairy root induction was obtained from kind courtesy of Dr. M. Banerjee professor, bau, kanke, ranchi.

Strain ATCC 15834 and MTCC 532 were used for genetic transformation and hairy root induction of *Withania somnifera.* YEB and YEN media were used for bacterial culture and growth of strain ATCC 15834 and MTCC 532 respectively. For culture of bacteria, solid media were used for respective bacteria and they were maintained at $28^0$ C and $25^0$ C respectively. Media devoid of agar was used for bacterial growth. Broth of bacteria was maintained at their respective temperature at 110 rpm and 140 rpm respectively.

**YEB Media (Used for Strain ATCC 15834)**

Beef Extract - 5.0gm/L

Peptone - 5.0gm/L

Yeast - 1.0gm/L

Sucrose - 5.0gm/L

$MgSO.7H_2O$ - 0.5gm/L

Agar - 15.0gm/L

PH - 7.0

**YEN Media (Used for Strain MTCC 532)**

beef extract - 1.0 gm/L

peptone - 5.0 gm/L

yeast - 2.0 gm/L

nacl - 5.0 gm/L

Agar - 15.0gm/L

PH - 7.2

**Media Used for Broth Were Devoid of Agar**

YEB liquid was used for bacterial growth (ATCC 15834). Broth was maintained at 110rpm at $28^0$ C for 48 hours. After 48 hours of culture, the broth was centrifuged at 6000 rpm for 10 minutes. YEN liquid was used for bacteria MTCC 532. The bacteria is temperature sensitive so bacteria must be maintained at $25^0$ C at 120 rpm for 24 hours. After 24 hours of culture the broth was centrifuged at 5000 rpm for 15 minutes Pellets obtained by centrifugation were acclimatized with MS liquid. Then MS liquid with bacterial pellets used for co-culture of sterile shoot tips. Shoot tips co-cultured for different time periods, viz. 15 mins, 30 mins, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 24hr. After particular co-culture period the shoot tips were washed properly with 250mg/L cefotaxime for 1 minute. After that the shoot tips were washed with autoclaved double distilled water for three times. The explants were then inoculated in MS basal medium without any hormones.

## RESULTS AND DISCUSSIONS

Various factors like type of explants (Dupre et al., 2000), *A.rhizogenes* strains (Giri et al., 2001; Tiwari et al., 2007) phenolic compounds (Kumar et al., 2006) growth medium (Azlan et al., 2002) bacterial concentration (John et al., 2009), growth hormones (Falasca et al., 2000) and pH (Danesh et al., 2006) showed great influence on root induction. The present study was conducted to check the efficacy of the two strains of *Agrobacterium rhizogenes* ATCC 15834 and MTCC 532 to induce hairy roots from shoot tips of *Withania somnifera.* Transformation frequency, number of hairy roots and number of lateral branches in roots were observed.

After 8-10 days, Proliferation of very small hairy roots were observed which continued to grow. ATCC 15834 gave 91.29 ± 1.75 % (Table 1) of maximum transformation frequency when explants were treated with 3 hours co-culture in contrast of MTCC 532 which showed 85.41 ± 2.08 % (Table 2) in 4 hours co-culture period. Both the strains showed gradual reduction in transformation frequency after attaining their maximum (Table 1 and 2). Pawar et al., 2003 found negative response when using stem segments of *Withania somnifera* using MTCC 532 and MTCC 2364. Chandran et al., 2007, reported hairy root induction through ATCC15834 *in Ipomoea batatas, Vigna vexillata*, and *Canavalia* sps.

**Table 1: Percentage of Transformation Frequency through Strain ATCC 15834**

| Time | 11 Days | 22 Days | 33 Days |
|---|---|---|---|
| control | - - | - - | - - |
| 15 mins | 08.51 ± 1.71 | 19.07 ± 1.86 | 24.43 ± 2.81 |
| 30 mins | 18.70 ± 1.77 | 32.77 ± 1.59 | 43.14 ± 1.63 |
| 1 hr | 30.74 ± 3.25 | 53.51 ± 2.23 | 64.25 ± 2.19 |
| 2hr | 43.88 ± 2.90 | 66.11 ± 2.01 | 79.62 ± 2.60 |
| 3hr | 55.18 ± 1.82 | 82.58 ± 2.02 | 91.29 ± 1.75 |
| 4hr | 38.70 ± 2.41 | 59.62 ± 1.66 | 68.51 ± 2.37 |
| 5hr | 24.07 ± 1.91 | 41.48 ± 0.93 | 53.51 ± 1.70 |
| 6hr | 11.85 ± 1.63 | 29.43 ± 2.01 | 37.78 ± 1.40 |
| 24hr | 07.03 ± 2.24 | 12.21 ± 1.57 | 22.78 ± 1.51 |

**Table 2: Percentage of Transformation Frequency through Strain MTCC 532**

| Time | 11 Days | 22 Days | 33 Days |
|---|---|---|---|
| Control | - - | - - | - - |
| 15 mins | 06.43 ± 2.15 | 10.60 ± 2.03 | 14.95 ± 2.23 |
| 30 mins | 15.33 ± 2.36 | 30.31 ± 1.74 | 36.92 ± 1.73 |
| 1 hr | 21.59 ± 1.96 | 34.65 ± 3.01 | 47.91 ± 2.79 |
| 2hr | 29.73 ± 2.12 | 46.77 ± 2.02 | 59.65 ± 3.64 |
| 3hr | 38.01 ± 1.53 | 58.70 ± 1.86 | 71.77 ± 1.78 |
| 4hr | 52.08 ± 2.07 | 74 99 ± 3.40 | 85.41 ± 2.08 |
| 5hr | 31.81 ± 1.51 | 53.21 ± 2.01 | 61.73 ± 2.04 |
| 6hr | 22.91 ± 2.09 | 38.33 ± 5.22 | 52.26 ± 5.20 |
| 24hr | 04.16 ± 2.40 | 08.33 ± 3.40 | 10.41 ± 2.08 |

Number of hairy roots induced were counted and 11.15 ±0.30 number of roots / explants after 33 days was observed in transformation through ATCC 15834 (Table 3 and Figure 1E) however 08.27 ± 0.35 roots/explants were achieved through MTCC 532 (Table 1 and Figure 2F). Akramian et al., 2008 achieved hairy roots in four sps of *Hyoscyamus* through ATCC 15834, 5.38 ±0.65 roots in *H. arachnoideus*, 6.63 ± 0.94 in *H. kurdicus*,. 6.13 ± 0.40 in *H. reticulates*, 6.25 ± 0.59 in *H. squarrosus*.

**Table 3: Mean Number ± SE of Roots/Explants in Transformation through Strain ATCC 15834**

| Time | 11 Days | 22 Days | 33 Days |
|---|---|---|---|
| 15 mins | 1.60 ± 0.24 | 1.83 ± 0.27 | 2.34 ± 0.16 |
| 30 mins | 2.83 ± 0.23 | 3.15 ± 0.10 | 4.13 ± 0.11 |
| 1 hr | 2.97 ± 0.13 | 4.02 ± 0.15 | 5.19 ± 0.21 |
| 2hr | 3.66 ± 0.14 | 6.16 ± 0.09 | 8.12 ±0.14 |
| 3hr | 5.75 ± 0.09 | 9.12 ± 0.08 | 11.15 ±0.30 |
| 4hr | 3.27 ± 0.13 | 4.48 ± 0.06 | 5.40 ±0.12 |
| 5hr | 2.08 ± 0.35 | 2.74 ± 0.17 | 3.41 ±0.10 |
| 6hr | 1.91 ± 0.20 | 2.41 ± 0.23 | 2.94 ± 0.19 |
| 24hr | 1.25 ± 0.25 | 1.58 ± 0.20 | 1.83 ± 0.21 |

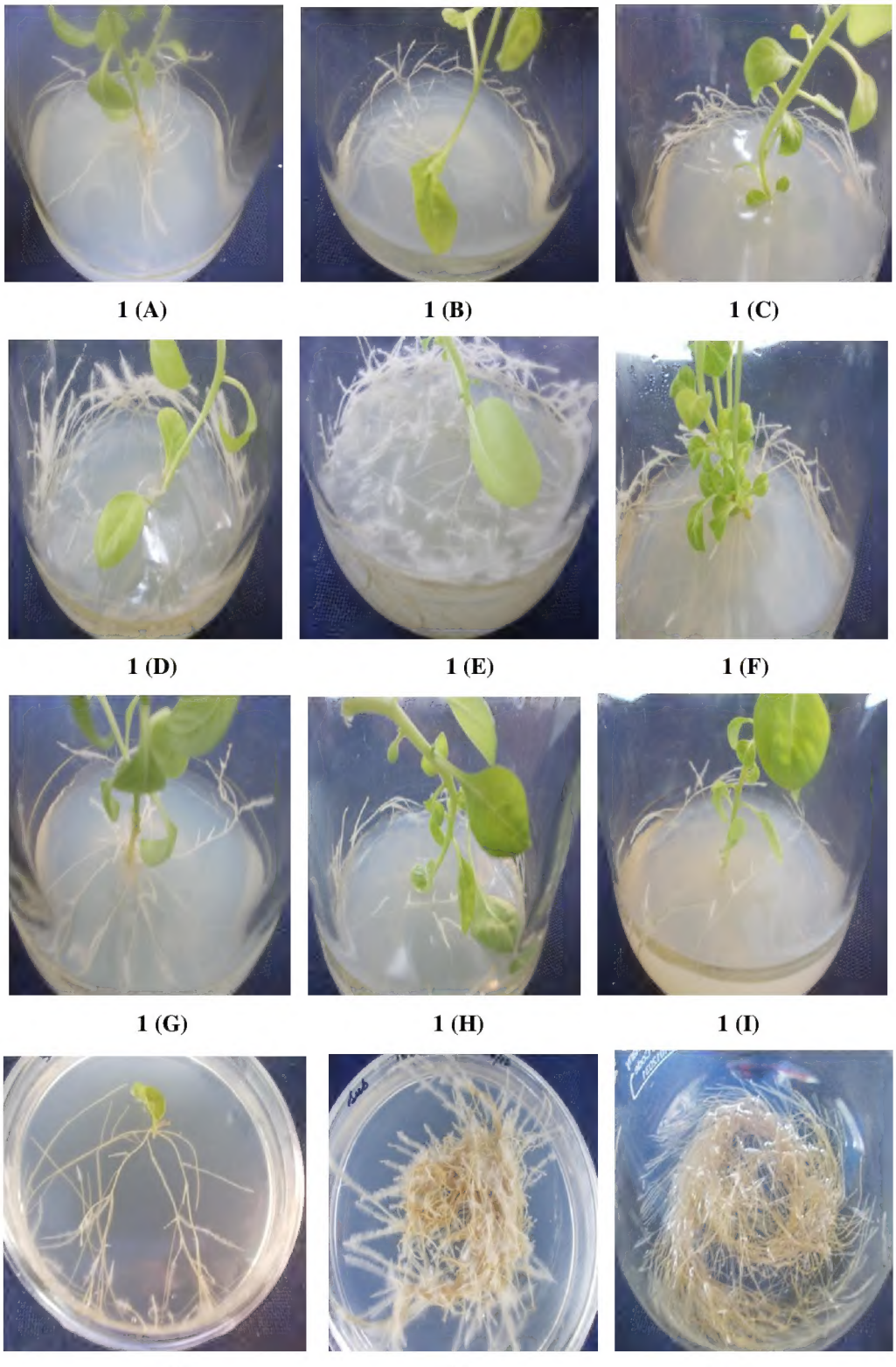
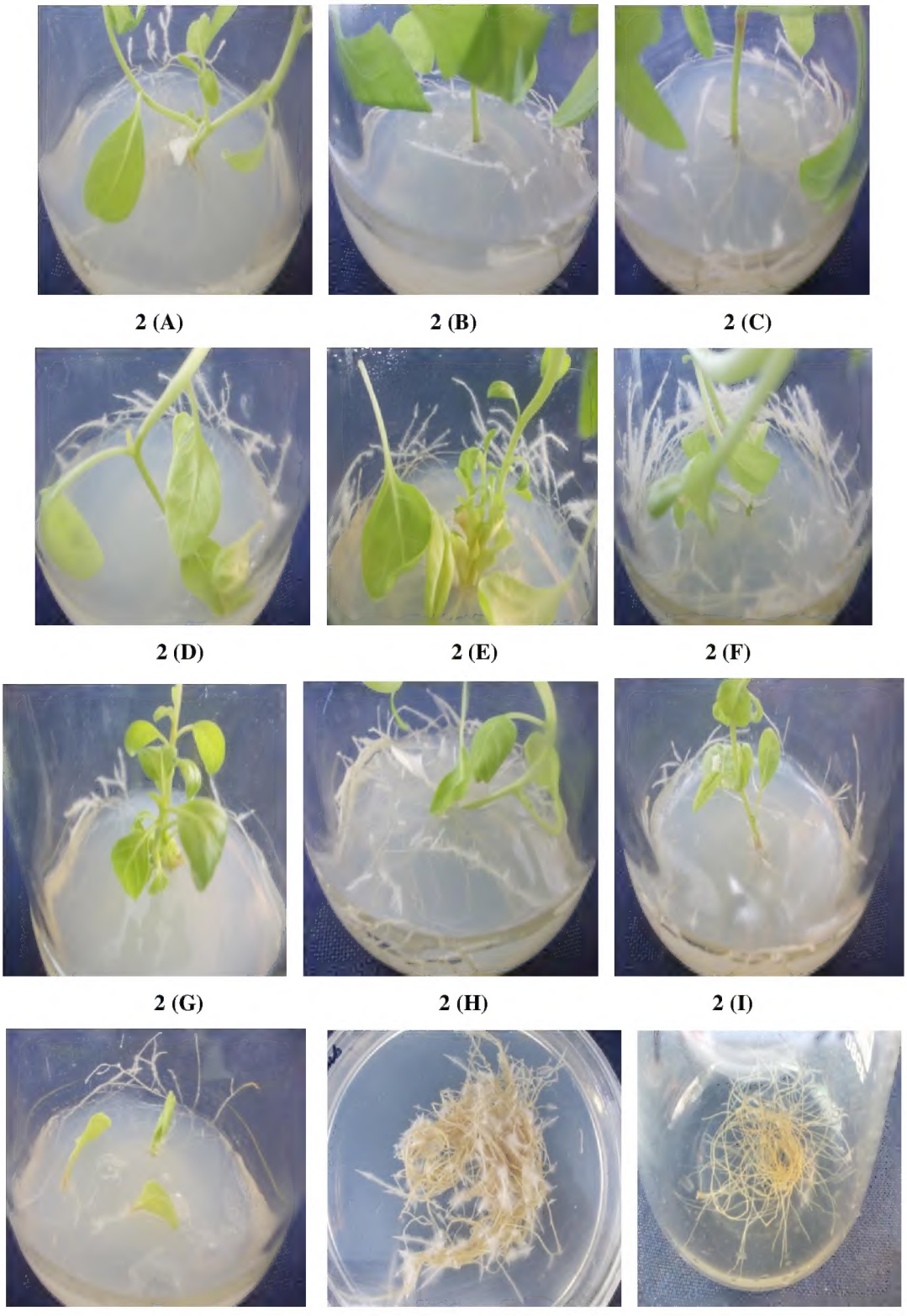



**Figure 1: A-L, Results Obtained through Strain ATCC15834 in Different Co-Culture Period viz 15 Mins, 30 Mins, 1 hr, 2hr, 3hr, 4hr, 5hr, 6hr and 24 hr (1A-I) 1J-Transformed Leaf to Root, 1K-Transformed Root to Root in Solid Media, 1L-Transformed Root to Root in Liquid Media**



2 (A) 2 (B) 2 (C)

2 (D) 2 (E) 2 (F)

2 (G) 2 (H) 2 (I)

2 (J) 2 (K) 2 (L)

**Figure 2: A-L, Results Obtained through Strain MTCC 532 in Different Co-Culture Period viz 15 Mins, 30 Mins, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr and 24 hr (1A-I) 1J-Transformed Leaf to Root, 1K- Transformed Root to Root in Solid Media, 1L-Transformed Root to Root in Liquid Media**

**Table 3: Mean Number ± SE of Roots/Explants in Transformation through Strain MTCC 532**

| Time | 11 Days | 22 Days | 33 Days |
|---|---|---|---|
| 15 mins | 01.25 ± 0.47 | 01.50 ± 0.28 | 01.87 ± 0.37 |
| 30 mins | 01.75 ± 0.25 | 02.12 ± 0.31 | 02.83 ± 0.39 |
| 1 hr | 01.96 ± 0.33 | 02.37 ± 0.14 | 03.05 ± 0.37 |
| 2hr | 02.49 ± 0.21 | 03.16 ± 0.13 | 04.24 ± 0.25 |
| 3hr | 02.91 ± 0.29 | 04.83 ± 0.09 | 06.13 ± 0.36 |
| 4hr | 03.67 ± 0.09 | 05.49 ± 0.17 | 08.27 ± 0.35 |
| 5hr | 03.23 ± 0.15 | 04.15 ± 0.14 | 05.95 ± 0.24 |
| 6hr | 02.37 ± 0.24 | 02.91 ± 0.22 | 03.35 ± 0.14 |
| 24hr | 0.75 ± 0.47 | 01.37 ± 0.62 | 01.87 ± 0.51 |

Lateral branching in roots were also counted. After 45 days 35.12 ± 0.23(Table 4 and Figure 2F) lateral branching in roots/explants were observed in transformation through MTCC 532 and 46.09 ± 0.16 lateral branching in roots/explants were recorded through strain ATCC15834 (Table 3 and Figure 2E).

**Table 4: Mean Number ± SE of Lateral Branching in Roots/Explants in Transformation through Strain ATCC 15834**

| Time | 15 Days | 30 Days | 45 Days |
|---|---|---|---|
| 15 mins | 3.40 ± 0.24 | 5.91 ± 0.15 | 9.25 ± 0.21 |
| 30 mins | 6.75 ± 0.22 | 12.70 ± 0.18 | 16.86 ± 0.15 |
| 1 hr | 8.30 ± 0.25 | 15.72 ± 0.17 | 20.07 ± 0.39 |
| 2hr | 11.51 ± 0.16 | 21.13 ± 0.44 | 34.84 ± 0.51 |
| 3hr | 17.87 ± 0.24 | 28.04 ± 0.21 | 46.09 ± 0.16 |
| 4hr | 13.99 ± 0.19 | 21.31 ± 0.12 | 31.83 ± 0.27 |
| 5hr | 6.08 ± 0.23 | 8.18 ± 0.30 | 12.47 ± 0.42 |
| 6hr | 4.75 ± 0.17 | 7.58 ± 0.15 | 11.83 ± 0.16 |
| 24hr | 2.50 ± 0.28 | 5.58 ± 0.20 | 8.16 ± 0.21 |

**Table 5: Mean Number ± SE of Lateral Branching in Roots/Explants in Transformation through Strain MTCC 532**

| Time | 15 Days | 30 Days | 45 Days |
|---|---|---|---|
| 15 mins | 02.25 ± 0.75 | 04.37 ± 0.24 | 06.12 ± 0.23 |
| 30 mins | 03.25 ± 0.47 | 05.37 ± 0.23 | 07.66 ± 0.45 |
| 1 hr | 04.79 ± 0.12 | 07 29 ± 0.17 | 09.28 ± 0.22 |
| 2hr | 06.24 ± 0.16 | 09.10 ± 0.14 | 12.19 ± 0.13 |
| 3hr | 07.12 ± 0.24 | 10.03 ± 0.15 | 15.33 ± 0.10 |
| 4hr | 13.45 ± 0.19 | 19.07 ± 0.37 | 35.12 ± 0.23 |
| 5hr | 10.79 ± 0.12 | 14.43 ± 0.07 | 21.40 ± 0.09 |
| 6hr | 04.12 ± 0.13 | 08.33 ± 0.31 | 13.54 ± 0.41 |
| 24hr | 01.75 ± 1.03 | 03.37 ± 1.14 | 05.86 ± 0.31 |

Soil born gram negative bacteria *Agrobacterium rhizogenes* are able to transfer apart of their DNA, T-DNA into the host genome which make them natural genetic engineer. Potential of hairy roots for production of secondary metabolites has been recognized since last two decades, Doran (1997). The main advantage of using hairy root cultures is their ability to grow in defined basal media without supplementation of phytohormones. The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules, Tao et al (2006). In the present study it was confirmed that different strains showed variation in their virulence even when same type of explants was used, it was supported by the previous works (Krolicka et al 2001, Zehra et al 1998, Vanhala et al 1995.)

## CONCLUSIONS

This study emphasises on production of hairy roots of *Withania somnifera* L. Dunal. From the above study it was confirmed that in between ATCC 15834 and MTCC 532 strain ATCC15834 showed maximum 91.29 ± 1.75 % of transformation frequency, 11.15 ±0.30 number of roots /explants and 46.09 ± 0.16 number of lateral branching in roots /explants in 3 hours of co-culture period in contrast of MTCC 532 which gave 85.41 ± 2.08 % of transformation frequency, 08.27 ± 0.35 number of roots /explants and 35.12 ± 0.23 number of lateral branching in roots /explants in 4 hours of co-culture period. After successful achievement of genetic transformation hairy roots induce directly from the sub cultured part of the plant (Leaf, Shoot tip, Stem segments and from the roots also) which provide a regular induction of hairy roots. The established hairy roots need to be further investigated for their secondary metabolites production. They may prove to be good source for large scale production of secondary metabolites in bioreactors.

## ACKNOWLEDGEMENTS

Talat Ara is thankful to DST, Government of India, for providing financial assistance in the form INSPIRE Fellowship.

## REFERENCES

1. Ahmad, M.S., Saleem, A.S., Ahmed, M.A., Ansari, S. Yousuf and Hoda, M.N. (2005). Neuroprotectine effect of *Wthania somnifera* on 6-hydroxydopamine induced parkin-sonism in rats. Hum and Exp. Toxicol. 24: 137 – 147.
2. Akramian, M., S. Mohammad, F. Tabatabaei a nd M. Mirmasoumi 2008.Virulence of Different Strains of *Agrobacterium rhizogenes* on Genetic Transformation of Four *Hyoscyamus* Species American-Eurasian J. Agric. & Environ. Sci., 3 (5): 759-763.
3. Archana Giri, Sarish T. Ravindra, Vikas Dhingra and M. Lakshmi Narasu 2001. Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua,* CURRENT SCIENCE, VOL. 81, NO. 4, 378-382.
4. Azlan GJ, Marziah M, Radzali M, Johari R (2002). Establishment of *Physalis minima* hairy roots culture for the production of Physalins. Plant Cell Tissue Org. Cult. 69: 271-278.
5. Bhattacharya, S.K., Satyan, K.S. and Chakarbarti, A. (1997a). Effect of Trasina, an Ayurvedic herbal formulation on paracentric is let superoxide dismutase activity in hyperglycamic rats. Ind. J. of Exp. Bio. 35: 297 – 299.
6. Chandran RP, VP Potty (2007) Induction of hairy roots through the mediation of four strains of *Agrobacterium rhizogenes* on five host plants. Indian J. of Biotechnol.7:122-128
7. Danesh YR, Mohammadi GE, Alizadeh A, Modarres SM (2006). Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. J. Biol. Sci. 6: 87-91.
8. Doran, P.M., 1997. Hairy Roots: Culture and Application. Harwood Academic Publishers, Amsterdam, Netherlands.
9. Dupre P, Lacoux J, Neutelings G, Mattar LD, Fliniaux MA, David A, Jacquin A (2000). Genetic transformation of *Ginkgo biloba* by *A.tumefaciens*. Physiol. Plant. 108: 413-419.

10. Falasca G, Reverberi M, Lauri P, Caboni E, Stradis A, Altamura MM (2000). How *Agrobacterium rhizogenes* triggers de novo root formation in a recalcitrant woody plant: an integrated histological, ultra structural and molecular analysis. New Phytol. 145: 77-93.

11. John KMM, Joshi SD, Mandal AKA, Kumar SR, Kumar RR (2009). *Agrobacterium rhizogenes*- mediated hairy root production in tea leaves [*Camellia sinensis* (L.) O. Kuntze}. Indian J. Biotechnol. 8: 430-434.

12. Giri A, Ravindra ST, Dhingra V, Narasu ML (2001). Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. Curr. Sci. 81: 378-382.

13. Królicka, A., I. Staniszewska, K. Bielawski,. E. Maliñski, J. Szafranek and E. Lojkowska, 2001. Establishment of hairy root cultures of *Ammi majus*. 79: 140-146 Plant Sci., 160: 259-264. 24.

14. Kumar V, Sharma A, Prasad NCB, Gururaj BH, Ravishankar AG (2006). *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. Electr. J. Biotechnol. 9: 349-357.

15. Pawar PK., VL Maheshwari (2004) Agrobacterium rhizogenes mediated hairy root induction in two medicinally important members of family solanaceae. Indian J. of Biotechnol. 3:414-417. S Rai, D.G., Bhatia, T. Sen and Palit, G (2003). Antistress effect of Ginkgo *biloba* and panax ginseng a comparative study Journal of Pharmacological Sciences 93: 458 – 64

16. Tao, J. and L. Li, 2006. Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes.* South African J. Bot., 72: 211-216.

17. Tiwari RK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007). Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep. 26: 199-210.

18. Vanhala, L., R. Hiltunen and K.M. Oksman- Caldentey, 1995. Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus.* Plant Cell Rep., 14: 236-240.

19. Zehra, M., S. Banerjee, A.A. Naqavi and S. Kumar, 1998. Variation in the growth and alkaloid production capability of the hairy root of *Hyoscyamus albus*, *H. muticus* and their somatic hybrid Plant Sci., 136: 93-99